Secreted Bacterial Outer Membrane Vesicles (OMVs) and their role in pathogenesis

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Certificate of Examination

This is to certify that the dissertation titled "Secreted Bacterial Outer Membrane Vesicles (OMVs) and their role in pathogenesis" submitted by Ms. Ashima Valentina Minj (Reg. No. MS10112) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

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(Supervisor)

Dated : November 2017

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Arunika Mukhopadhyay at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, diploma or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Ashima Valentina Minj

(Candidate)

Dated : November 30, 2017

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Arunika Mukhopadhyay

(Supervisor)

Acknowledgement

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Ashima Valentina Minj

Contents

Abstract

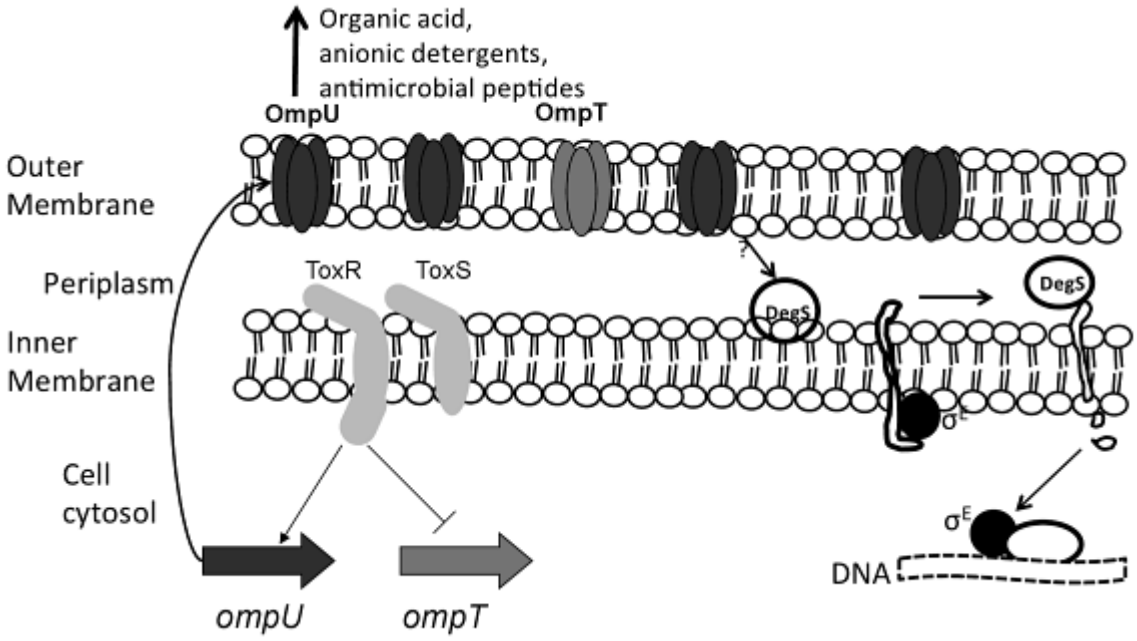
OMVs are spherical extracellular secretions for proteins and lipids of gram-negative bacteria with Variety of functional roles of OMVs important for baceria growth survival and proliferation. OmpU in *Vibrio cholerae* confers a lot of properties like Bile salt resistance ,Resistance against anti-microbial peptides and in Survival and colonization in the gut . The Objective was to Characterize the role of secreted bacterial OMVs in pathogenesis . It was found that In cells treated with rOmpU , rOmpU gets translocated to the mitochondria but gets degraded 4 hours post treatment.But vesicle gets translocated to the mitochondria of the host cell and OmpU is seen to be present intact uptil 24 hrs post-treatment . hence it is Hypothesized that Intact vesicle gets translocated to the mitochondria of the host cell and OmpU gets prevented from degradation by the protective vesicle environment .

INTRODUCTION

Basic Theory

Outer membrane vesicles (OMVs) are small closed spheroid particles (~10 t0 300 nm diameter) released from gram-negative bacteria and a few gram-positive bacteria. They are produced when a portion of the outer membrane bulges with the subsequent fission of vesicles containing periplasmic content. OMVs are distinct from membranous blebs produced during cell lysis as they are produced as a regulated , selective secretion event. Unlike other secretion mechanisms, OMVs enable bacteria to secrete insoluble molecules in addition to and in complex with soluble material. Rates of OMV production are not uniform , even for a particular strain, as production has long been observed to be influenced by environmental factors and by sources of cellular stress. In general, OMV reflects OM composition, containing Lipid polysaccharides, glycerophospholipids , and OM proteins as well as enclosed periplasmic contents. The virulence factors of gram-negative pathogens are secreted products that enhance the survival of the bacteria and/or damage the host. The secretion of virulence factors by Type IV Mechanism is important as smaller and non-viable, so it can influence an environment that is inaccessible to the whole bacterium. OMVs can disseminate far from the cell and impart biological functions on the environment and other cells, including playing a role in pathogenesis, quorum signalling, nutrient acquisition and horizontal gene transfer. Analysis of naturally produced OMVs from pathogenic bacteria have demonstrated presence of wide variety of virulence factors which include protein adhesins, toxins, enzymes and non-protein antigen like LPS and other immunomodulatory compounds , and they directly mediate bacterial binding and invasion, cause cytotoxicity and modulate host immune response. By participating in such diverse aspects host-pathogen interaction, OMVs are potent bacterial virulence factors.

OmpU which is outermembrane protein is an acidic protein which is homotrimer of 38-kDa monomeric subunits and is a porin with pore size 1.6 nm. Its secondary structure contains predominantly a β-sheet and three to four Ca^{2+} ions are associated with each monomeric unit. This *tox*R-regulated protein is a virulence determinant. *Tox*R is an important regulator of the outer membrane stress response protein and has been shown to respond to external bile concentration and increases the expression of OmpU. Presence or absence of OmpU determined a strain's sensitivity to anionic detergents, much in the same way the ability to produce OmpU played a role in strain's ability to mount an organic acid adapted response. Apart from being important for survival of bacteria in the presence of bile, OmpU also plays a role in stress response to organic acids and antimicrobial peptides.



OmpV is a 25-kDa heat-induced, highly immunogenic protein associated with peptidoglycan that may be related to high salt resistance. The ability in salt-tolerance was reduced in the cells overexpressed OmpV. V. paraheamolyticus mutants without OmpV could recover the response when they were complemented by *Photobacterium damsela* OmpV. In *P. damsela* , it's been seen that there's abundant OmpV at low osmolarity. However, information regarding the ability of salt regulation for the protein remains elusive and its characterization needs to be done.

1.1 Experimental Methods and Techniques

SDS-PAGE

Stands for sodium dodecyl sulphate - Polyacrylamide gel electrophoresis. a technique widely used in to separate proteins according to their electrophoretic mobility. For proteins, sodium dodecyl sulfate (SDS) is an anionic detergent applied to protein sample to unstructure proteins into linear chains and to impart a negative charge to linearized proteins. In most proteins, the binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis.

Materials required

- Solution A (30% acrylamide solution)

- for 100ml solution 29.2g Acrylamide + 0.8g N'N'-bis-methylene- acrylamide in deionized (MilliQ) water

- Solution B (Lower Tris Buffer) 4X

  - 1.5M Tris (18.17 g Tris base)+ 4.0 ml 10% SDS. Adjusted pH to 8.8. Diluted to 100mL with deionized water

- Solution C (Upper Tris Buffer) 4X)

-0.5 M Tris (6.06 g Tris base) +4.0 ml 10% SDS. adjusted pH to 6.8. Diluted to 100mL with deionized water

- Running buffer
  -14.4g glycine + 3g Tris base + 1g SDS . make volume upto 1000mL.

- Loading dye 5X
  -3.9 ml deionized water + 1.0 ml 0.5 M Tris, pH 6.8 + 0.8 ml Glycerol + 1.6ml 10% SDS + 0.4 ml 2-β mercaptoethanol + 0.4 ml 1% bromophenol blue

- TEMED (tetramethylethylenediamine)

- Ammonium persulfate

- Staining solution : Dissolve 0.25 g of Coomasie brilliant blue in 45 ml of methanol. Add 45 mL of H2O and 10 ml of acetic acid

- Destaining solution : 80mL H2O + 15mL methanol + 5mL acetic acid

Western blot

Western blotting is an important technique used in cell and molecular biology. By using a western blot, researchers are able to identify specific proteins from a complex mixture of proteins extracted from cells. In this technique a mixture of proteins is separated based on molecular weight, and thus by type, through gel electrophoresis. These results are then transferred to a membrane producing a band for each protein. The membrane is then incubated with labels antibodies specific to the protein of interest.

Material Required:

- 10X TBS (Tris-buffered saline) pH- 7.6 :

  Tris Base 24.2g + NaCl 80g +ddH2O to 1L.

- 1X TBST (Tris-buffered saline in tween20) :

  10x TBS 100ml + ddH2O 900ml + Tween-20 1ml

- Transfer Buffer :

  Tris Base (25mM) 3.03g + Glycine (192mM) 14.4g + ddH2O 800ml + Methanol 200ml

- Blocking solution : 5% Bovine serum albumin (BSA) in TBST

An intact SDS PAGE electrophoresis system should include: a tank, lid with power cables, electrode assembly, cell buffer dam, casting stands, casting frames, combs(usually 10-well or 15-well), and glass plates (thickness 0.75mm or 1.0mm or 1.5mm).

PROTOCOL

Casting the gel

- Before and after experiment clean both notch plate and glass p la t e
  With water followed by 70% Alcohol. wipe it with Kim
  wipe or air dry it use for experiment(don't use detergents and tape water for washing)

- Clean and completely dry the glass plates, combs, and any other pertinent materials.
- Place a short plate on top of a spacer plate. Insert both plates into the green casting frame on a flat surface. Be sure that the "legs" of the casting frame are down. Clamp the casting frame and check that the plates are level on the bottom.
- Put the casting frame into the casting stand.

10% SDS PAGE Cocktail

Sr No.	10% Separating Gel (ml)	Stacking gel (ml)
H2O (HPLC grade)	4	1.36
30% Acryl amide	3.3	0.34
Tris	2.5(1.5M)	0.25(0.5M)
10% SDS	0.1	0.02
10% APS	0.1	0.02
TEMED	0.004	0.002
Total volume (ml)	10	2

- Combine all reagents except the TEMED for the 10% separating gel.
- W hen ready to pour the gel, quickly add the TEMED, mix using a Pasteur pipette, and transfer the separating gel solution between the glass plates in the casting chamber to about 3/4 inch below the short plate.
- A small layer of Distilled Water can be added on top of the gel prior to polymerization to straighten the level of the gel and remove unwanted air bubbles that may be present. Once the gel has polymerized, the water is removed by absorption

with Kimwipes or filter paper. Rinse the top layer of the gel with dI water prior to pouring the stacking gel.

- Combine all reagents except the TEMED for the 5% stacking gel.
- When ready to pour the gel, quickly add the TEMED, mix using a Pasteur pipette, and transfer the stacking gel solution between the glass plates in the casting chamber.
- Insert the well forming comb into the opening between the glass plates.
- Let the stacking gel polymerize.
- Once the stacking gel has polymerized, the comb can be gently removed. The polymerized gel between the short plate and spacer plate forms the "gel cassette".

Sample Preparation

- Determine the protein concentration for each cell lysate by Bradford Regent method.
- Place some water in a 600 mL beaker and leave on a hot plate to boil(usually 100˚C)
- Meanwhile, add 50 mL of 2-mercaptoethanol to 950 mL of sample buffer.
- Determine the protein concentration for
- Take 20 µg of each sample and add an equal volume of 2x sample buffer
- Boil each cell lysate in sample buffer at 95°C for 5 min.Centrifuge at 16,000 x g in a microcentrifuge for 1 min

Electrophoresis

- Remove the gel cassette from the casting stand and place it in the electrode assembly with the short plate on the inside.
- Slide the electrode assembly (with the gel cassette) into the clamping frame. Press down on the electrode assembly while clamping the frame to secure the electrode assembly. This step is important to minimize potential leakage during the electrophoresis experiment.
- Pour some 1X electrophoresis buffer into the opening of the casting frame between the gel cassettes. Add enough buffer to fill the wells of the gel. Use a gel loading tip to pipette some buffer into each well to ensure cleanliness.

- Load equal amounts of protein (20 μg) into the wells of a PAGE gel, along with molecular weight markers.
- Fill the region outside of the frame with 1X electrophoresis buffer.
- Cover the tank with the lid aligning the electrodes (black or red) appropriately.
- Connect the electrophoresis tank to the power supply.
- Run the gel for 5 min at 50 V.
- Increase the voltage to 100–150 V to finish the run in about 1 hr until the dye front reaches the bottom of the gel.
- When electrophoresis is complete, turn off the power supply and disassemble the apparatus.

For Western blotting

- Run the gel as you would for SDS-PAGE.
- Place the gel in 1x transfer buffer for 10–15 min..
- Assemble the transfer sandwich and make sure no air bubbles are trapped in the sandwich. The blot should be on the cathode and the gel on the anode.
- Place the cassette in the transfer tank and place an ice block in the tank.
- Transfer overnight in a cold room at a constant current of 10 mA.
- Transfer can also be done at 100 V for 30 min–2 h .

Antibody incubation

- Briefly rinse the blot in water
- Block in 3% BSA in TBST at room temperature for 1 hr.
- Incubate overnight in the primary antibody solution, against the target protein, at 4°C.

(Note:The antibody should be diluted in the blocking buffer according to the manufacturer's recommended ratio. Primary antibody may be applied to the blot for 1–3 hr at room temperature depending on antibody quality and performance.)

- Rinse the blot 3–5 times for 5 min with TBST.
- Incubate in the HRP-conjugated secondary antibody solution for 1 hr at room temperature.
- Rinse the blot 3–5 times for 5 min with TBST.

EXPERIMENTAL PROCEDURE

Now that the fundamental methods to be used for the experiment have been explained along with the protocol. We proceed with the experiment.

GROWTH KINETICS

Before isolation of vesicles, we had do the growth kinetics for the organisms under investigation. This is because it is a well established fact that maximum vesiculation occur during the late log phase in most microorganisms.

Requirements:

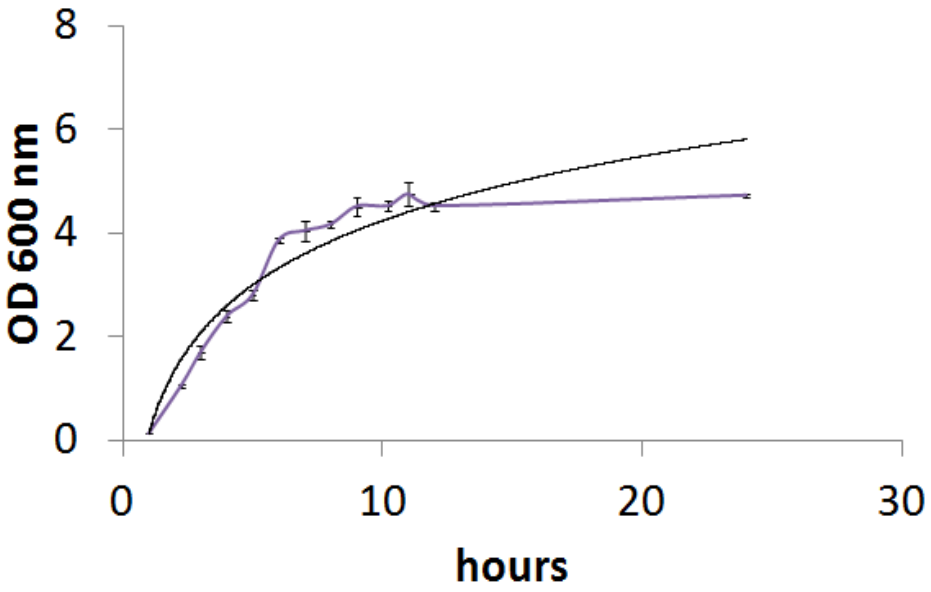
LB-Agar plate, laminal hood, 70%EtOh, burner, conical flasks, Conical flask, 50 mL Luria Bertini media (for inoculation) + 20 mL (for dilution), cuvette, spectrophotometer , pipette

Procedure:

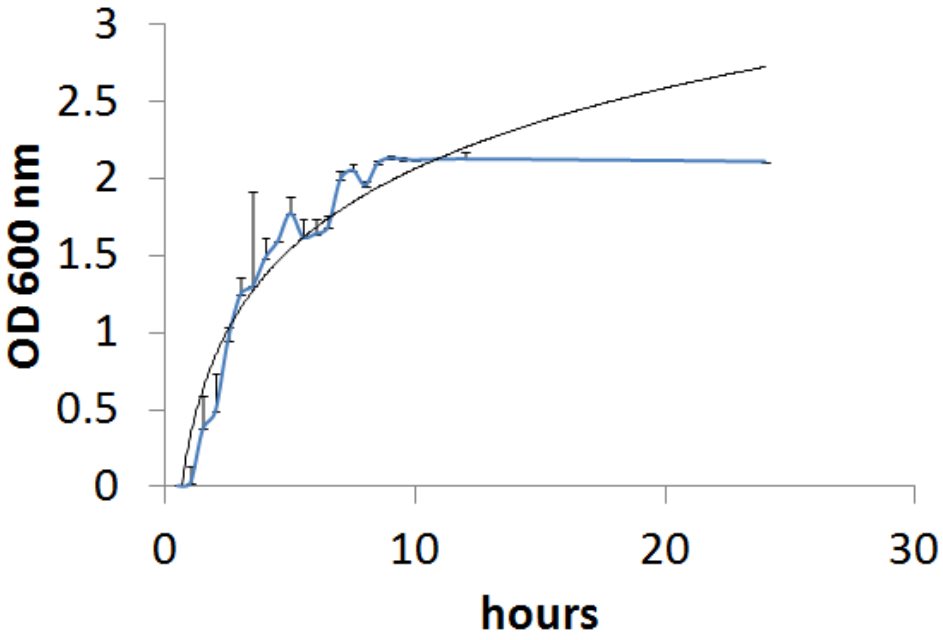
- From the seed culture stored in -80˚C we did streaking on Luria Broth-Agar plates and kept it in 37˚C overnight to obtain single colonies. Next day the plates were checked for growth of single colonies and then the plates were stored in 4˚C.
- One of the colonies were picked and inoculated in 3mL media and kept in 37˚C overnight
- Next day The Optical density at 600nm for the overnight inoculated culture was checked and calculation was done to find out how much of the culture was to be put in 50mL LB media so that OD is 0.01 and kept in 37˚C
- Optical density at 600 nm was taken every 1 hour post inoculation until we started getting stationary phase / not much fluctuating readings for the inoculated media.
- It was made sure that OD Remained below 0.4 , if the OD exceeded the mark , dilutions and subsequent calculation for final OD were made
- All the readings were noted and plotted to establish the late-log phase for the bacterium.

RESULTS:

GROWTH KINTICS FOR ***Vibrio cholerae***



GROWTH KINETICS FOR ***Salmonella typhimurium***



GROWTH KINETICS FOR ***Vibrio parahaemolyticus***

To summarize;

Sample microorganism	Late log phase (hrs)
Vibrio cholera	~ 9
Salmonella typhimurium	~8
Vibrio parahaemolyticus	~9

Vesicles Isolation :

Requirements

2 L LB-Media, bacterial stock, swing buckets, conical flasks, oakridges, centrifugation machine, ampicillin, sodium azide, EDTA, 20 Mm Tris, Microcentrifuge tubes (MCTs), autoclaved bottles, ultracentrifuge tubes, ultracentrifugation machine.

Procedure

FOR OUTERMEMBRANE VESICLE ISOLATION

- Inoculate one of the single bacterial colony in about 30 mL media and let it grow for 8-9 hrs. Afterwards inoculate 10% of the culture in the 1 L LB media batches which are to be used for isolation. And keep in 37˚C.
- At the designated time for the late log phase (about 8 to 9 hrs) for the corresponding bacterium , transfer the culture in swing buckets and balance the weights.
- Low- speed Centrifuge at 5000^{x}g for about 30 mins. At 4˚C to break the cells.
- Transfer the supernatant in autoclaved bottles and Discard the pellets . For storing the supernatant add 0.05% sodium azide, 1mM EDTA and 1% penicillin .
- To clear off the debris from the supernatant, centrifugation at 12,000 rpm for 30 mins at 4˚C . Store the supernatant and discard the pellet obtained.
- Supernatant after second round of centrifugation was subjected to ultracentrifugation at $150,000^{x}$g for 2hrs at 4˚C .
- Discard the supernatant and continue ultracentrifugation until all the supernatant is exhausted.
- Resuspend the pellet in about 6-7mL or as per the how dense the pellet is,in 20 mM Tris , do the washing by ultracentrifugating at $150,000^{x}$g for 30mins. at 4˚C and discard the supernatant. Repeat this step two more times.
- Finally after the washing is over , resuspend the pellet in 1mL 20mM Tris. Aliquot 100µL pellet in 10 MCTs and store in -20˚C.

FOR OUTER MEMBRANE ISOLATION

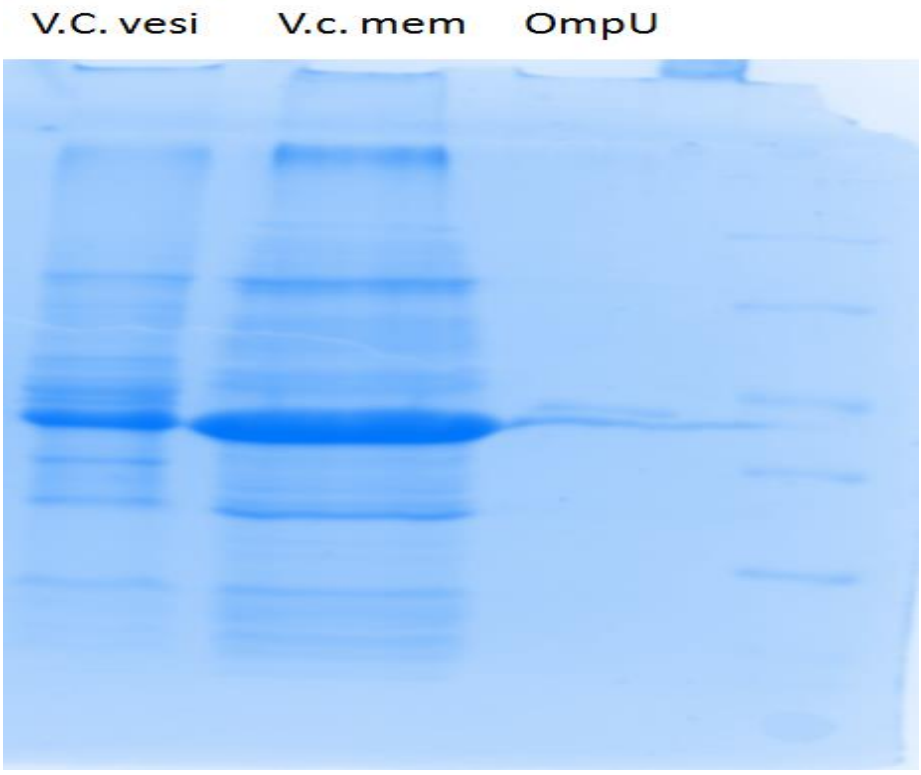
- Inoculate 10% of the cell culture in 1L batch and keep in 37˚C for 9 to 10 hrs.
- Transfer the cell culture to the swing buckets and balance . Do low-speed centrifugation at 6000Xg for 30 mins. at 4˚C.
- Discard the supernatant and resuspend the pellets in 10mL HEPES Buffer (pH- 7.4) and add 50µL of bacterial protease inhibitor.
- Sonicate the pellet solution for 15 mins. at 25 amplitude for every 20 ses with 10 sec break.
- The membranes were collected by ultracentrifugation at 100 000Xg for 1 h at 4 °C.
- The pellet was resuspended in 2 ml 10 mM HEPES (pH 7.4).
- Then the pellet suspension was spun again in the ultracentrifuge at 100 000Xg for 1 h at 4 °C.
- The pellet was resuspended in 5 ml 1 % (w/v) *N*-lauroylsarcosine in 10 mM HEPES Buffer (pH 7.4) and incubated at 37 °C for 30 min with shaking.
- The Sarkosyl-treated membranes were spun at 100 000Xg for 1 h at 4 °C.
- The pellet washed with 10 ml 10 mM HEPES, pH 7.4 and final ultracentrifugation was done at 100 000Xg for 1 h at 4 °C.
- Following the final ultracentrifugation, the pellet was resuspended in 500 µl 10 mM HEPES, pH 7.4.

SDS- PAGE

For establishing the difference between OMV's and Outermembrane of bacteria

RESULT

1. Protein profile for ***Vibrio Cholerae***



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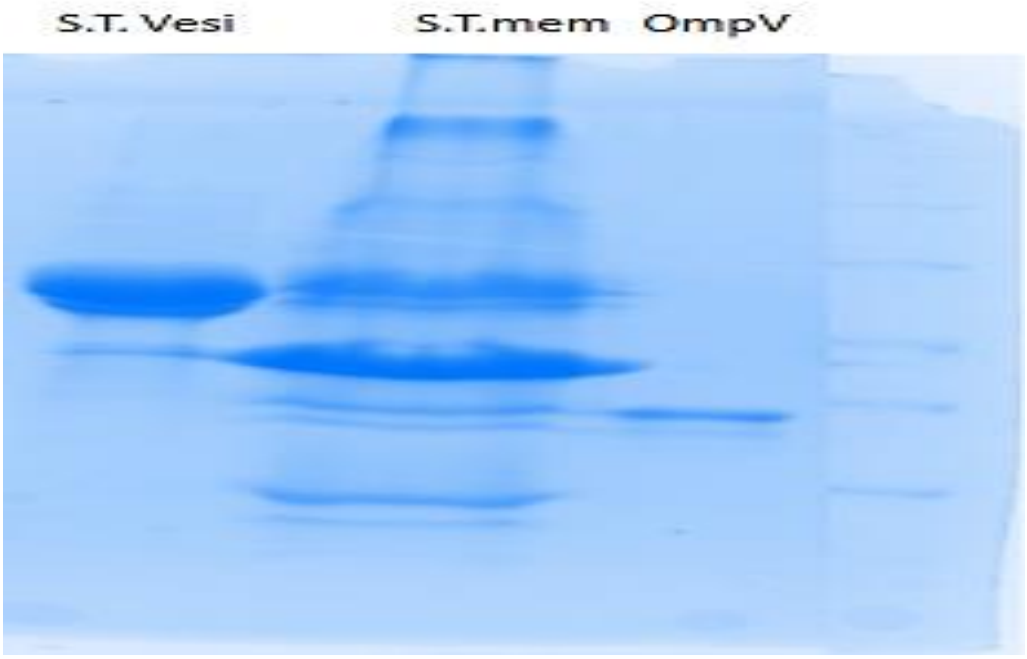
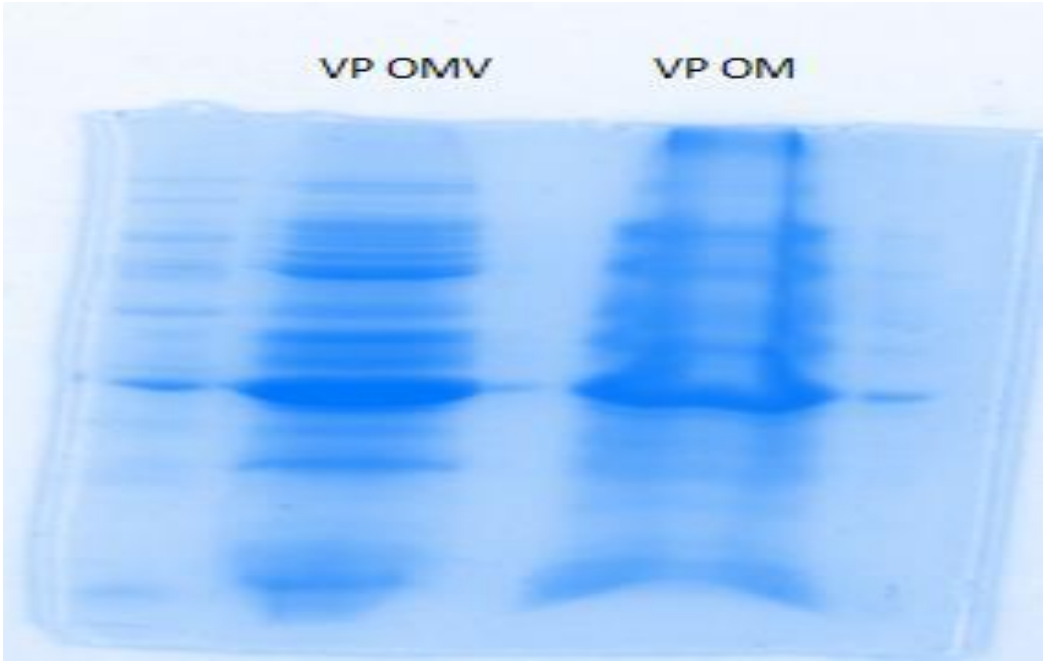
2. Protein profile for ***Salmonella Typhimurium***



3. Protein profile for ***Vibrio Parahaemolyticus***

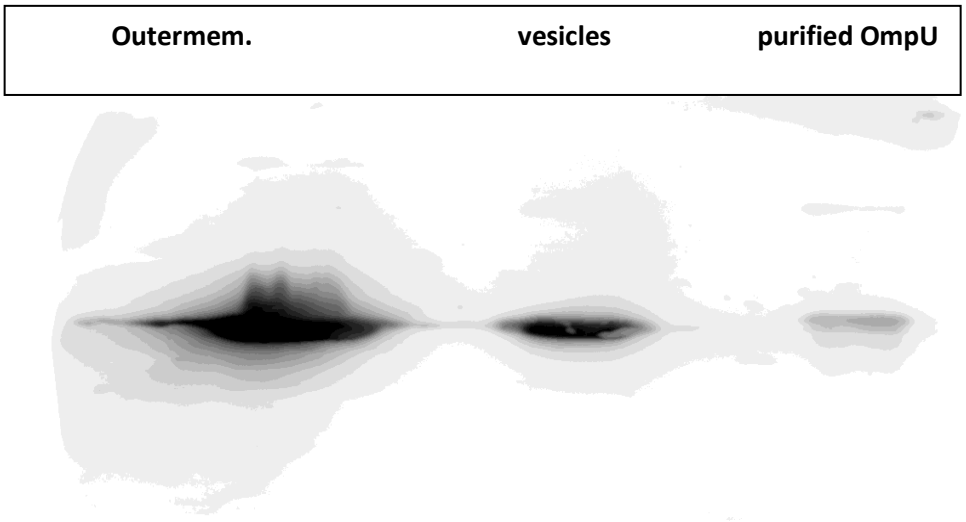


Western blot analysis

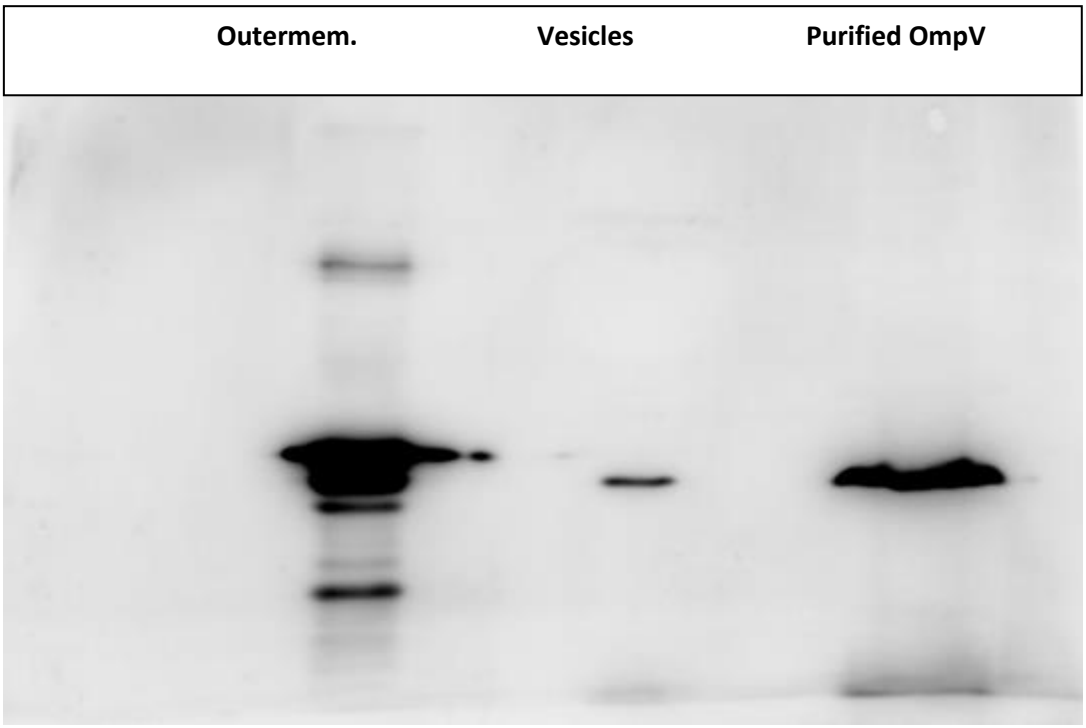
For the detection of the proteins of interest and Confirmation for presence of Omps in OMVs by Assaying for OmpU in the isolated *V. cholerae* vesicles and OmpV in *Salmonella typhimurium* vesicles by western blot analysis . we did not further go ahead further probing for *Vibrio parahaemolyticus* as the protein of interest OmpU has very low expression in vesicles.

RESULT

OmpU probing for *Vibrio cholerae*



OmpV probing for *Salmonella typhimurium*

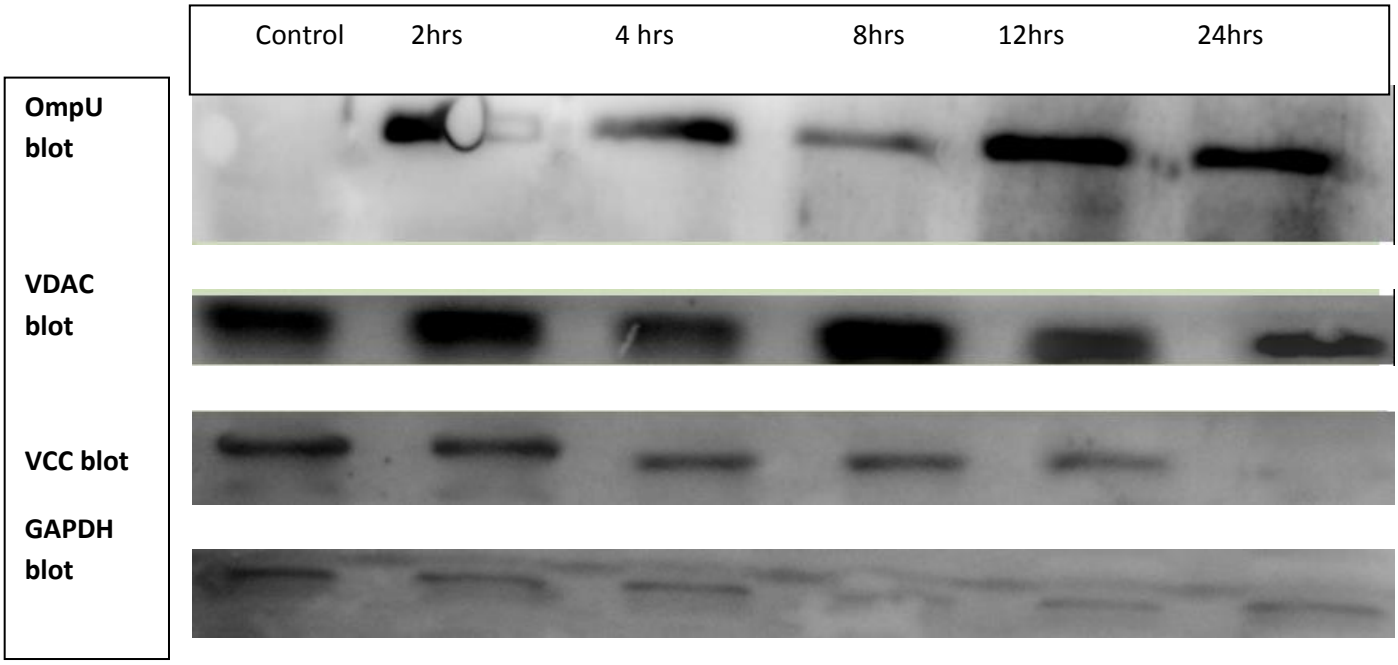


Translocation of OmpU to mitochondria

- Thp1 cell lines were treated with vesicles isolated from *V. cholerae* , mitochondria from treated cells isolated and probed for OmpU . Since the Protein OmpV was present in low concentration in the vesicles experiments on Salmonella typhimurium were discontinued.
- It was seen earlier in experiments done the lab that in cells treated with rOmpU , rOmpU gets translocated to the mitochondria but gets degraded 4 hours post treatment

RESULT

For *Vibrio Cholerae*



Proteins Used-

VDAC- mitochondrial proteins controlling cell life and death is the **voltage-dependent anion channel (VDAC)**, also known as mitochondrial porin. VDAC, located in the mitochondrial outer membrane, functions as gatekeeper for the entry and exit of mitochondrial metabolites, thereby controlling cross-talk between mitochondria and the rest of the cell. VDAC is also a key player in mitochondria-mediated apoptosis.

VCC - The enteric pathogen *Vibrio cholerae* secretes a water-soluble 80-kD cytolysin, ***Vibrio cholerae*** **cytolysin (VCC)** that assembles into pentameric channels following proteolytic activation by exogenous proteases. it is present in outermembrane .

GAPDH- Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) a multifunctional enzyme with both glyceraldehyde-3-phosphate dehydrogenase and nitrosylase activities. Is cytosolic and is present in high levels in all cells and is often used as a protein normalizing control.

Summary and Conclusion

Result

In cells treated with rOmpU , rOmpU gets translocated to the mitochondria but gets degraded 4 hours post treatment..But vesicle gets translocated to the mitochondria of the host cell and OmpU is seen to be present intact uptil 24 hrs post-treatment

Conclusion

Intact vesicle gets translocated to the mitochondria of the host cell and OmpU gets prevented from degrading by the protection provided by vesicle

Future Directions

- Check the hypothesis by probing with anti-LPS Ab
- Study the pathway about vesicle mediated pro-inflammatory response (receptors and cytokines released involved)

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